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High-hydrostatic-pressure optical chamber system for cultivation and microscopic observation of deep-sea organisms

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ABSTRACT: We developed and tested a high-pressure optical chamber system for cultivation and microscopic observation of deep-sea organisms. The system is composed of an optical chamber, a high-pressure pump, a pressure sensor and a microscope. The chamber has an observation cavity, 2 cultivation cavities and 2 sapphire windows. The pump is employed for perfusion of culture medium and for increasing the pressure. The pressure sensor monitors the pressure in the chamber. The microscope is used for observing samples through the sapphire windows. In the future, the optical chamber system could be used alone for short-term research or be connected to a large high-pressure vessel to create a flow-through system for long-term research. Using the system, the swimming activity of *Bosmina longirostris* (Branchiopoda: Cladocera) was observed at different pressures. Swimming activity increased with increasing compression up to 30 MPa. During decompression, this activity reappeared when pressure decreased to 45 MPa and increased with further decreasing pressure.

KEY WORDS: Optical chamber system · Real-time · Microscopic observation · Plankton

INTRODUCTION

Many organisms live in the deep sea at high pressure and low temperatures. Since large numbers of biological communities were first discovered near hydrothermal vents (Lonsdale 1977), much interest has been focused on the effects of hydrostatic pressure on a variety of deep-sea organisms (Sakiyama & Ohwada 1998, Kaye & Baross 2004, Partridge et al. 2006, Campanaro et al. 2008). Many studies have shown that organisms retrieved from depths of 2000 to 3000 m or more do not survive under normal pressure (Gross & Jaenicke 1994, Pradillon et al. 2004, Pradillon & Gaill 2007). The effect of hydrostatic pressure on non-deep-sea organisms has been examined as well (Castillo et al. 2004, Black et al. 2005, Abe 2007, Horikawa et al. 2009).

In previous studies of biological systems, most experiments were based on the examination of the effect of pressure treatment by comparing states before and after the treatment. However, the lack of real-time microscopic observations of organisms, such as meiofauna, greatly weakens the conclusions of pressure effects. Many apparatuses have been designed for cultivation and real-time observation of organisms under high hydrostatic pressure (Yancey 2009). The hydrostatic pressure of these apparatuses ranges from 0.1 to 300 atm (Fraser & Macdonald 1994, Marsh et al. 2001, Fraser & Shelmerdine 2002, Pradillon et al. 2004, Shillito et al. 2004). Some apparatuses have windows of sapphire or Pyrex glass for real-time observation (Gregg et al. 1994, Grasset 2001, Koyama et al. 2001, Raber et al. 2006). However, these apparatuses have some drawbacks (Frey et al. 2006, Oger et al. 2006): (1) The sample volume is small and excludes some multicellular organisms; (2) Long-term real-time microscopic observations are not feasible; (3) The pressure is not high enough for deep-sea (e.g. 6000 m depth) organisms; (4) Pressure chambers are made of stainless steel, which may influence the organisms and can be damaged by seawater.

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To overcome these drawbacks, we developed a high-pressure optical chamber system specifically used for cultivation and microscopic observation of organisms ranging from 100 to 1000 µm in size. The high-pressure chamber is made of titanium, which has been proven to be an excellent biocompatible material and can be used under high pressure up to 60 MPa (6000 m depth in the deep sea). The chamber can either be used alone for short-term research or as part of a flow-through system by connection with a large vessel. A flow-through system could maintain water chemistry conditions in the pressure chamber for long-term observation without decreasing the pressure. We examined the stability of the pressure chamber system and successfully made real-time observations of plankton under different pressures.

MATERIALS AND METHODS

Optical chamber system. The high-hydrostatic-pressure optical chamber system is mainly composed of an optical pressure chamber, a microscope, a pressure pump and a pressure sensor. The temperature is controlled by a water bath.

High-hydrostatic-pressure optical chamber: The optical chamber (Fig. 1) is made of titanium, an excellent material for high-pressure research. The chamber is in the form of a rectangular box (180 × 44 × 38 mm), which has an observation cavity and 2 cultivation cavities. The maximum pressure in the chamber is 60 MPa.

The observation cavity (Fig. 2a) is 13 mm in diameter and provides 2 opposite optical windows for real-time microscopic observation. The optical window (Fig. 2b) is made of sapphire, 16 mm in diameter and 8 mm in thickness. It provides clear images with common microscopes and excellent biocompatibility. A fluorine rubber O-ring (16 mm diameter, 1.8 mm thick) is used to seal the window. The sapphire window is tightened by a compression cover, which has coned holes for microscopic observation. The 2 cultivation cavities, both 18 mm in diameter and 72 mm in length, are located at the 2 sides of the observation cavity. These cavities are connected to the observation cavity by channels of 3 mm in diameter and 6 mm in length.

The chamber has 2 pipe connections. For short-term observation, samples are injected into the chamber directly and cultivated for several days. In this case, only one pipe connection is used. For long-term observation, the chamber is connected to a large high-pressure vessel to built up a real-time flowing observation system. In this case, both pipe connections are used, one for inflow and the other for outflow. A net is used to prevent organisms from being flushed from the system during circulation.

Microscope and pressure pump: The pressure chamber is compatibe with a variety of microscopes by using objective lenses with long working distance. It is also feasible to use 2 kinds of pump for altering pressure: a hand pump for fast alteration and a reciprocating

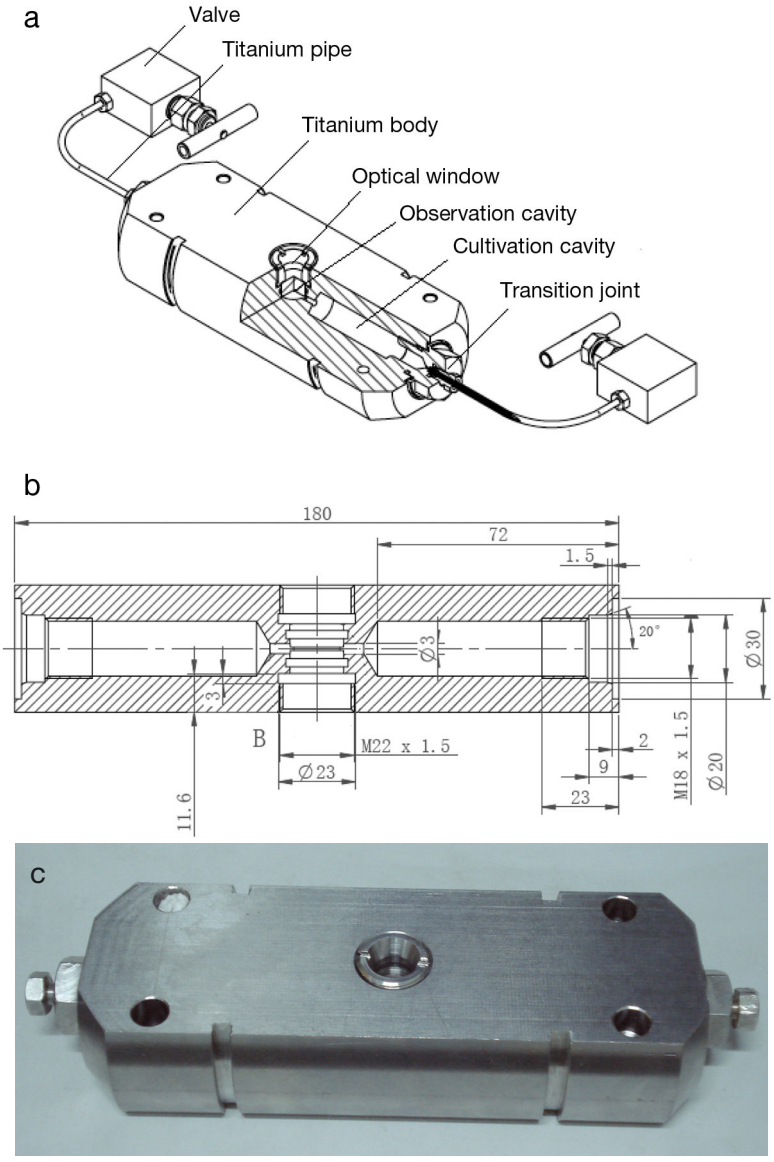

Fig. 1. (a) Schematic, (b) scale-drawing and (unit: mm) (c) photograph of the high-hydrostatic-pressure optical chamber, which includes an observation cavity, 2 cultivation cavities and 2 sapphire windows

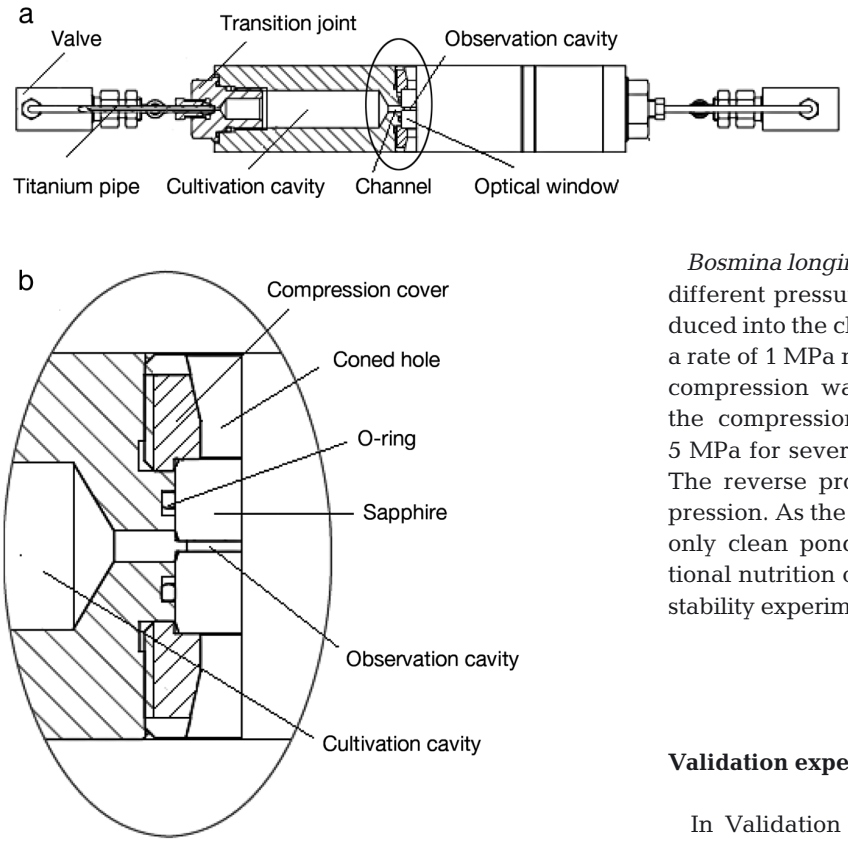

Fig. 2. (a) A longitudinal section of the high-hydrostatic-pressure optical chamber and (b) a close-up of the optical window which is circled in Fig. 2a

pump for slow alteration. The pressure in the chamber is monitored by a pressure sensor.

Study organisms. *Cyclops vicinus* (Cyclopoida: Crustacea) and *Bosmina longirostris* (Branchiopoda: Cladocera) were collected from a shallow freshwater pond and then transferred to an aquarium at room temperature. They were observed closely and found to be swimming very actively.

The organisms were observed with a long distance microscope (XTB-01) and the images were recorded via a CCD camera (Sony DSC-W50). Pressure was increased by a hand pump (JB-80) and monitored by a pressure sensor (pressure up to 80 MPa).

Validation experiments and *Bosmina* observations. Two types of validation experiments were performed to examine the pressure stability of the pressure chamber. In the first (Validation Expt 1), pressure was increased to 60 MPa at different compression rates (1, 2, 5 and 10 MPa min^{-1}) and maintained for 2 h. In the second (Validation Expt 2), pressure was increased to either 10, 20, 30, 40, 50 or 60 MPa at the rate of 1 MPa min^{-1} and maintained for 2 h. To examine the imaging performance of the system, pictures of *Cyclops vicinus* in the pressure chamber were taken with the camera mounted on the microscope. These were compared with images of the copepod on a common glass slide.

Bosmina longirostris were observed in real-time under different pressures. Several *B. longirostris* were introduced into the chamber. The pressure was increased at a rate of 1 MPa min^{-1}. When the pressure was 0.2 MPa, compression was stopped for several minutes. Then the compression was continued and stopped every 5 MPa for several minutes until 60 MPa was reached. The reverse process was carried out during decompression. As the experiments lasted only several hours, only clean pond water was used, without any additional nutrition or aeration. The *Bosmina* trials and the stability experiments were repeated twice (3 replicates).

RESULTS

Validation experiments of the high-pressure chamber

In Validation Expt 1 (pressure stability at 60 MPa using different compression rates), fluctuations of pressures occurred mainly in the first 30 min once maximum pressure was reached, and the maximal fluctuation was 2.9 MPa (Fig. 3).

In Validation Expt 2 (pressure stability at different target pressures using a compression rate of 1 MPa min^{-1}), the ratios (P1:P0; P0 represents the target pressure and P1 represents pressure after 2 h) were between 0.95 and 0.96, indicating that once target pressures were reached, the pressures were stable during the 2 h observation period (Fig. 4).

The pictures taken of *Cyclops vicinus* in the pressure chamber were clear enough to observe activity in the samples, though darker and slightly blurred compared to photographs of *C. vicinus* on a common glass slide (Fig. 5).

Real-time observation of the behavior of *Bosmina longirostris* at different pressures

Before compression, the organisms were distributed along the observation cavity and displayed slight swimming activity. During the course of compression to 0.2 MPa, there was an immediate and distinct increase in swimming activity. When the pressure was stable at 0.2 MPa, the swimming activity declined to a level lower than that before the previous stable pressure. This

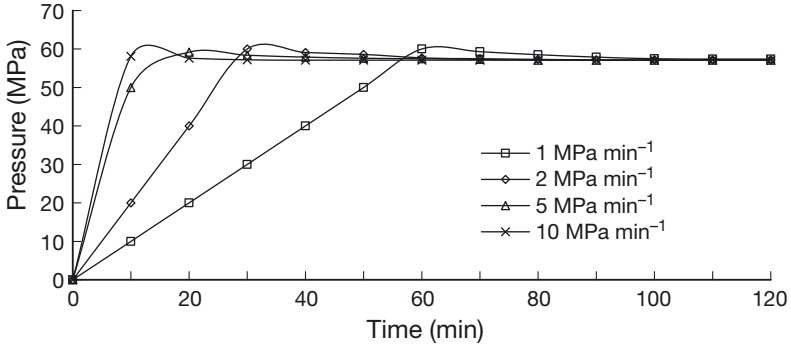

Fig. 3. Pressure fluctuations during compression from 0 to 60 MPa at different compression rates and while maintaining maximum pressure (60 MPa) for 2 h (Validation Expt 1)

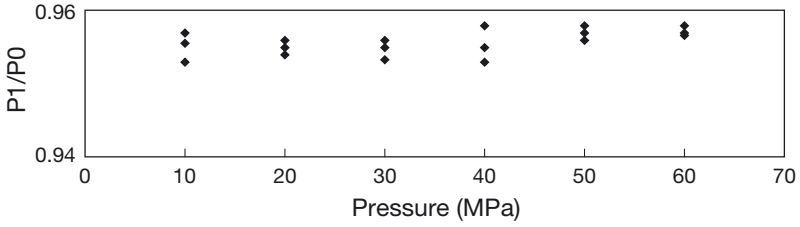

Fig. 4. Pressure fluctuations at different target pressures maintained for 2 h (Validation Expt 2). Fluctuations are expressed by the ratio P1:P0 where pressure after 2 h (P1) is divided by the target pressure (P0). The compression rate was 1 MPa min^{-1}

phenomenon appeared repeatedly until 30 MPa. When pressure was between 30 and 45 MPa, there was no swimming activity except for leg vibrations. When the pressure was above 45 MPa, even the leg vibration stopped.

During the course of decompression, swimming activity occurred again at 45 MPa and increased slowly as the pressure decreased below 45 MPa. This activity indicated that *Bosmina longirostris* survived exposure to high pressure of up to 60 MPa,. These results are similar to earlier reports (Forward & Wellins 1989, Forward 1990), but the real-time observation of *B. longirostris* under such high pressure has not been studied before.

DISCUSSION

In our experiments, a hand pump was used to increase pressure, as reported in other studies (Ross & Quetin 1985, Pradillon et al. 2004), but this method exposes

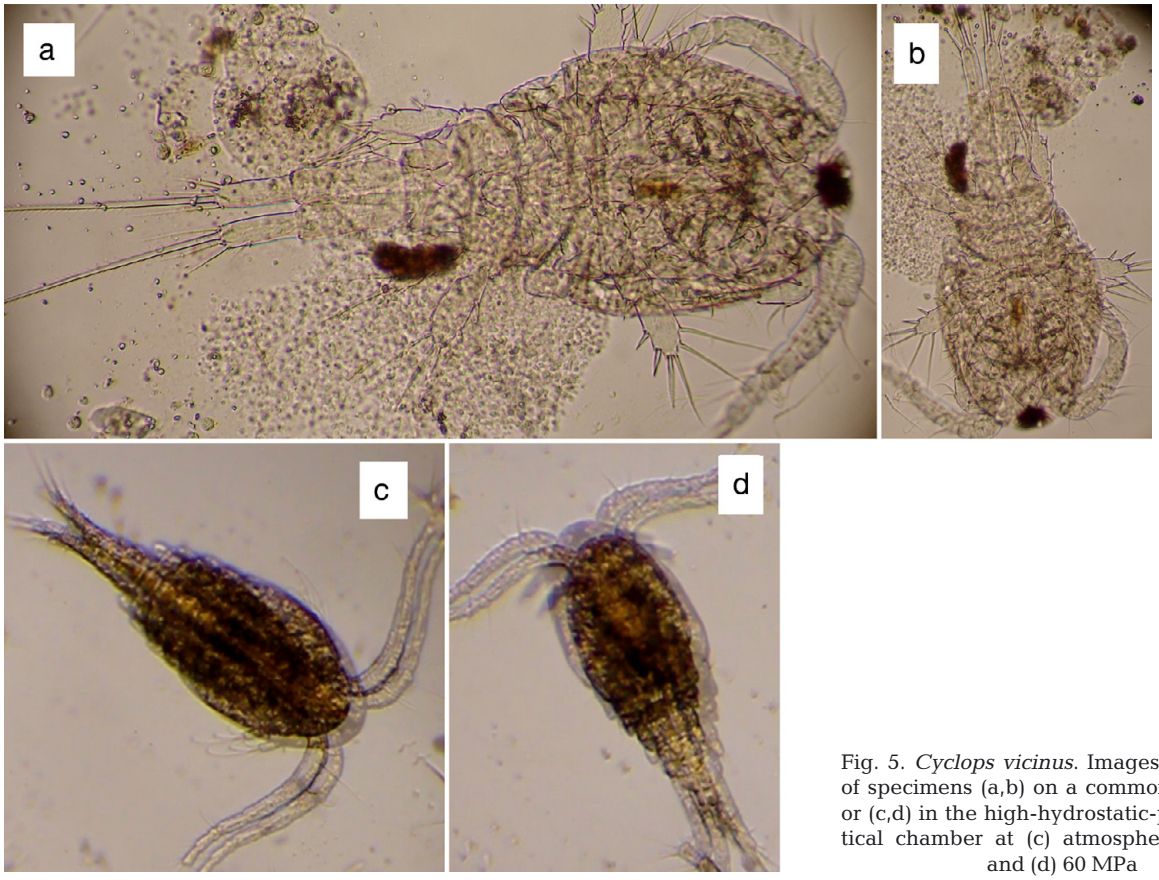

Fig. 5. *Cyclops vicinus*. Images were taken of specimens (a,b) on a common glass slide or (c,d) in the high-hydrostatic-pressure optical chamber at (c) atmospheric pressure and (d) 60 MPa

organisms to abruptly increasing pressure, which may destroy the structure or change the characteristics of samples. Therefore, some researchers propose a reciprocating pump instead (Yoshiki et al. 2006). The reciprocating pump compresses the medium smoothly at a constant flow rate and maintains pressure more accurately in the chamber. We made a tie-in for the purpose of connecting a reciprocating pump. This improves the applicability of the high-pressure optical system (Bao et al. 2010).

Many zooplankton live throughout the water column and are exposed to a range of hydrostatic pressures in their lifetime. *Bosmina longirostris* belongs to the Branchiopoda, an important component of planktonic crustaceans. Many researchers have investigated the effects of hydrostatic pressure on the behavior of zooplankton (Childress & Thuesen 1993, Bailey et al. 1994, Yoshiki et al. 2006, Yoshiki et al. 2008), but there is no direct evidence of the effects of extra-high pressure on the behavior of freshwater planktonic crustaceans. The high-hydrostatic-pressure optical chamber system allowed real-time observation and provided direct observations of biology under high pressure in our study of the behavior of *Bosmina longirostris* under different pressures.

In these experiments, we found that the high-hydrostatic-pressure optical chamber offered slightly darker and more blurred images compared to photographing the organism on a glass slide through a microscope. This might be caused by the intensity of light in the chamber and the thickness of the observation window. It could be improved by enhancing the light intensity or using a fluorescence microscope.

CONCLUSIONS

The high-hydrostatic-pressure chamber system was specifically used for microscopic observation. The chamber system provided a means of microscopic observation of organisms under extreme high pressure of up to 60 MPa. The results showed that the pressure chamber system was stable for real-time observation under high pressure. The chamber could be used conveniently with different microscopes. It also provides video and still images if a camera is installed. Using the system, *Bosmina longirostris* was directly observed under high pressures.

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